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Journal of Microbial Biology
E-ISSN: 3060-7647
13rd Year, Vol. 13, No. 52, Winter 2024 pp. 55-62
Received: 18/05/2024 Accepted: 13/08/2024

(Research Paper)

Influence of ellagic acid on oxidative stress tolerance and *rpoS* gene expression in clinical isolates of *Pseudomonas aeruginosa*

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Abstract
Due to the emergence of antibiotic-resistant clinical strains of Pseudomonas aeruginosa, there is an urgent need for a suitable and affordable alternative that is a non-antibiotic antimicrobial agent and creates a new generation of infectious disease treatment. In this regard, this study aims to investigate the antimicrobial potential of ellagic acid against clinical strains of P. aeruginosa under physiological growth conditions and oxidative stress. P. aeruginosa isolates from clinical samples were identified by biochemical tests and their antibiotic susceptibility was tested by disc diffusion method. The inhibitory effect of ellagic acid against multiple drug resistance isolates was investigated by disc diffusion and broth microdilution methods and its effect on bacterial survival under physiological conditions and oxidative stress was investigated by Time-Kill assay. The effect of ellagic acid on rpoS gene expression was also investigated by the Real time-PCR method. In this study, ellagic acid showed an inhibitory effect on the growth of all MDR isolates of P. aeruginosa tested. The minimum inhibitory concentration (MIC) of ellagic acid in 5 isolates varied between 250 and 2000 µg/ml. Treatment with ellagic acid rendered drug-resistant clinical strains of P. aeruginosa sensitive to oxidative stress. It reduced the survival of P. aeruginosa, while treatment with 1/4MIC concentration of ellagic acid resulted in a 4.7-fold decrease in log10 CFU/mL compared to the control. In the present study, treatment with a sub-inhibitory concentration of ellagic acid also caused a significant decrease in rpoS gene expression ($P<0.05$). The results of this study indicate that ellagic acid inhibits the growth of P. aeruginosa and increases its sensitivity to oxidative stress conditions. Conducting in vivo studies may clarify the possibility of its clinical application in the control of infections caused by resistant isolates of P. aeruginosa.

Keywords: Pseudomonas aeruginosa, Ellagic acid, oxidative stress, rpoS

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Taghinejad, P., & Asadpour, L. Influence of ellagic acid on oxidative stress tolerance and rpoS gene expression in clinical isolates of Pseudomonas aeruginosa. Journal of Microbial Biology. 2024; 13 (52): 55-62. http://dx.doi.org/10.22108/bjm.2024.141497.1592

Introduction

Pseudomonas aeruginosa is the causative agent of community and hospital-acquired infections ranging from serious systemic infections, endocarditis and ventilator-associated pneumonia to the relatively common urinary tract infections. This bacterium successfully causes infection in immunocompromised individuals, mechanically ventilated patients, the elderly or those with a history of cystic fibrosis, often leading to lung failure (1). In particular, multidrug-resistant (MDR) *P. aeruginosa*, which causes more than 50,000 healthcare-associated infections annually in the United States, is an emerging threat to human health. The pathogenicity and success of *P. aeruginosa* as a human and environmental pathogen is due to several factors, including its ability to develop the mechanism of acquired resistance and its intrinsic resistance mechanism to several classes of antibiotics and disinfectants (2). Although the antibiotics used against the microorganisms that cause infections are active in the laboratory, they may not have similar effects in the patient's body. The activity of antibiotics and antimicrobial compounds in vitro does not always reflect the same activity in vivo. Significant physiological changes occur in bacteria depending on various environmental factors such as thermal shock, food starvation, hydrogen peroxide pressure, high osmotic pressure and growth phase (3). Despite its ability to adapt to different environmental conditions, *P. aeruginosa* is constantly exposed to oxidative stress in its natural environment, whether endogenously produced by respiration or exogenously produced by the host immune system or disinfectants (2). These environmental conditions can induce genes that respond to stress. It has previously been reported that bacterial cells in the stationary phase are more resistant to antibiotics than those in the logarithmic growth phase. One reason for this is their slow growth rate. It has been proposed that stationary phase cells express different stress response genes that allow them to survive harsh environmental conditions. The antibiotic resistance of stationary phase cells may be influenced by genes controlled by the alternative sigma factor, RpoS. This factor has been identified in several Gram-negative bacteria, including *P. aeruginosa*, and the *rpoS* gene encodes this sigma factor. RpoS positively regulates many genes in stationary phase and is an important response regulator in bacteria (3). It is also involved in the protection of bacteria against oxidative, osmotic, acidic and thermal stress, adaptation to nutrient-limited conditions and the production of virulence factors (4).

Natural plants are a rich source of many compounds that fight drug-resistant bacteria. Ellagic acid is a phenolic acid widely found in fruits and nuts and has several biological activities, including antioxidant, antibacterial, antiviral, anti-inflammatory, anticancer, neuroprotective and anti-diabetic effects (5,6). It has been suggested that ellagic acid enhances the activity of current antimalarial drugs such as chloroquine, mefloquine and atovaquone (7). Ellagic acid has been shown to have potent anti-proliferative activity against colon, breast and prostatic cancer cell lines at concentrations in the range of 10-100 µmol/L. However at a similar dose, it did not affect the viability of normal fibroblast cells during a 24-hour period (8). Furthermore, in a 90-day toxicity study of ellagic acid at doses ranging from 9.4 to 42.3 g/kg of body weight, no deaths or treatment-related changes in clinical signs were observed in rats, and a toxic dose of ellagic acid was significantly higher than normal dietary levels (9). In humans, a maximum concentration of ellagic acid was detected in plasma after one hour of ingestion of pomegranate juice, but was rapidly eliminated after four hours. In the intestine, ellagic acid is metabolized by the gut flora to produce urolithins, which are much more bioavailable and enter the circulation within a few hours of ingestion, reaching peak concentrations between 24 and 48 hours (10).

Considering the clinical importance of *P. aeruginosa* and the high adaptability of this bacterium to environmental stress, this study was conducted to investigate the effect of ellagic acid on adaptability to oxidative stress and *rpo*S gene expression in clinical isolates of *P. aeruginosa*.

Materials and Methods

Test Bacteria

A total of 20 clinical isolates of *P. aeruginosa* were isolated from clinical skin and burn samples in Rasht. Various biochemical tests were performed to confirm the diagnosis of the bacteria, including the ability to produce haemolysin, oxidase, catalase, urease, ability to grow at 42 °C, growth pattern on McConkey agar, TSI, SIM and Mueller Hinton agar media. Colony and bacterial morphology and Gram staining were also used to detect test bacteria (11). The antibiotic resistance pattern of *P. aeruginosa* isolates was determined by disc diffusion according to CLSI guidelines (12). Antibiotic discs including amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefoxitin (30 µg), imipenem (10 µg), meropenem (10 µg), piperacillin (100 µg), erythromycin (30 µg), azithromycin (30 µg), cotrimoxazole (25 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg) and colistin (5 µg), purchased from High Media-India,

http://dx.doi.org/10.22108/bjm.2023.137161.1532

were used to screen for MDR *P. aeruginosa* strains.

Investigation of the inhibitory effect of ellagic acid against *P. aeruginosa*

Disc Diffusion Method

The disc diffusion method was used to investigate the antimicrobial effect of ellagic acid on MDR clinical isolates of *P. aeruginosa*. For this purpose, 100 µl of standard microbial suspension ($1.5x10^7$ CFU/ml) was cultured on Mueller-Hinton agar and discs impregnated with 5 mg ellagic acid were transferred to the culture. The culture was incubated for 24 hours at 37°C. The plate was then inspected and the diameter of the inhibition zone measured. This experiment was carried out in 3 replicates (13).

Determination of Minimum Inhibitory Concentration (MIC)

To determine the MIC of ellagic acid on *P. aeruginosa* isolates, two-fold serial dilutions of ellagic acid (4000-1 µg/ml) were prepared in Muller Hinton broth in a microtitre plate. Then 100 µl of standard microbial suspension ($1.5x10^5$ CFU/ml) of selected *P. aeruginosa* isolates were added to each well. Then 100 µl of the resulting microbial suspension was added to each well and the plates were incubated at 37°C for 24 hours. This experiment was performed in 3 replicates (14).

Time-Kill Assay

The efficacy of ellagic acid against clinical isolates of *P. aeruginosa* was determined using the kill time calculation method at various time intervals (0, 2, 4, 8 and 24 hours). In this assay, 100 µl of a bacterial suspension of $5x10^5$ CFU/ml was cultured in Mueller-Hinton broth containing 2MIC, MIC and 1/2MIC concentrations of ellagic acid and incubated at 37°C. At time intervals (0, 2, 4, 8 and 24 hours) of incubation, 100 µl of culture from each tube was removed, serially diluted (1:10) with PBS, plated onto Mueller-Hinton agar and incubated at 37°C for 24 hours. The number of viable bacteria was measured for each treatment. The time-kill assay was performed in duplicate (15).

Investigation of the effect of ellagic acid on oxidative stress tolerance

The fresh culture of selected isolates of *P. aeruginosa* was grown aerobically at 37^0C for 18 h. Then 100 µl of a suspension of 5×10^5 CFU/ml stationary phase bacteria was exposed to oxidative stress (30 mmol/L H_2O_2) for 30 min at 37°C. After neutralization of H_2O_2, organisms were treated with 1/2 MIC and 1/4 MIC concentrations of ellagic acid and the survival of the treated bacteria compared to the control group stressed with 30mM H_2O_2 and in the absence of ellagic acid was calculated and compared at 1, 2, 4 and 6 hours (16).

Investigation of the effect of ellagic acid on the expression of *rpo*S

Test isolates **were** treated with **a** sub-MIC concentration (500 µg/ml) of ellagic acid for 24 hours and RNA extraction was performed using an RNA extraction kit (sinalclon, Iran) according to the manufacturer's protocol. In addition, bacterial culture in the absence of antimicrobial substances was used as **a** control. cDNA was synthesized from the extracted RNA using random hexamer primers according to a commercial kit protocol (Thermofisher Scientific Inc.). The synthesized cDNA was then used as a template in a Real-time PCR reaction and amplified using SYBR green master mix (Ampliqon, Denmark). In this study, the *rpsL* housekeeping gene was used as a standard gene (17). The specific primers for the genes studied are listed in Table 1. The polymerase chain reaction was performed in a volume of 20 µl using the kit from Genet bioCAT. NO: Q9210 (South Korea) according to the kit instructions. The change in gene expression was calculated by $2^{-\Delta\Delta CT}$ (18). The experiment was repeated three times.

Table 1. Names and nucleotide sequences of the primers used in the Real-time PCR reaction

Gene Name	Nucleotide Sequence (´5 to ´3)	Tm	Product Length (base pairs)	Reference
rpoS-F	CTCCCCGGGCAACTCCAAAAG	63	198	7
rpoS-R	CGATCATCCGCTTCCGACCAG			
*rps*L-F	GCAACTATCAACCAGCTGGTG	60	231	
*rps*L -R	GCTGTGCTCTTGCAGGTTGTG			

Statistical analysis

Statistical analyses were performed using Student's t-test, and $P \leq 0.05$ was considered significant.

Results

Based on biochemical characteristics, a total of 20 *P. aeruginosa* isolates were recovered during the study period. Based on antibiotic susceptibility testing, the highest phenotypic resistance was to erythromycin (75%), and colistin (100% susceptible) was the most effective antibiotic. Resistance to other antibiotics tested included

amikacin (35%), gentamicin (30%), ceftazidime (55%), cefotaxime (50%), ceftriaxone (40%), cefoxitin (45%), imipenem (15%), meropenem (25%), piperacillin (45%), azithromycin (70%), cotrimoxazole (65%), ciprofloxacin (65%), enrofloxacin (75%). Of these 60% of isolates (n = 12) had a multidrug-resistant (MDR) phenotype (resistant to three classes of antibiotics) and 25% (n = 5) were resistant to three classes of antibiotics plus imipenem. These isolates were selected for further investigation.

Inhibitory effect of ellagic acid on clinical isolates of *P. aeruginosa*

The effect of ellagic acid on the inhibition of growth of *P. aeruginosa* isolates was investigated by disc diffusion method and MIC determination (Table 2). The diameter of the zone of inhibition caused by 1 mg ellagic acid in the test isolates varied between 8 - 25 mm (Figure 1). The MIC values of ellagic acid in 5 isolates varied between 250 - 2000 µg/ml.

Table 2. Diameter of inhibition zone and MIC of ellagic acid in *P. aeruginosa* isolates

Test Bacteria	**1**	**2**	**3**	**4**	**5**
Zone of inhibition (mm)	8	14	15	17	25
MIC (µg/ml)	2000	1000	1000	500	250

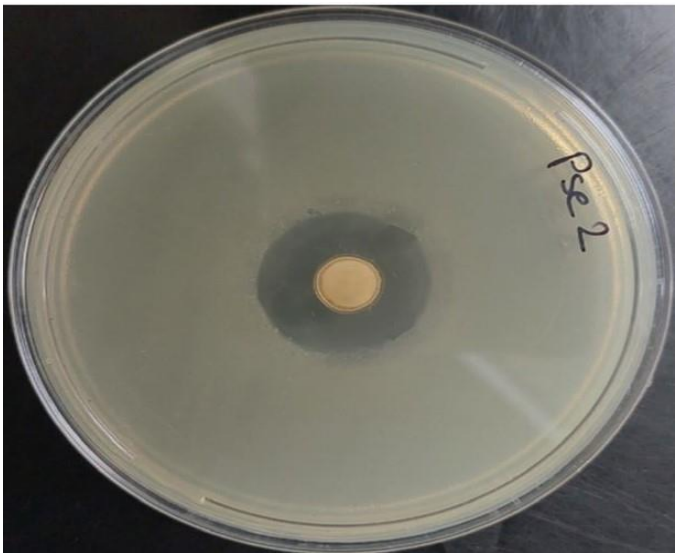

Figure **1**. The inhibitory effect of ellagic acid (10 mg) on *P. aeruginosa* using the disc diffusion method.

Time killing assay

The bactericidal activity of ellagic acid against selected isolates of *P. aeruginosa* as measured by,changes in log10 CFU/mL of live cells, is shown in Figure **2.** In the control, log **10** CFU/mL reached 11.5 after 24 hours of incubation at **37**°C. When bacteria were treated with a concentration of **1/2** MIC of ellagic acid, a gradual decrease in the number of bacteria was observed between 0 and 24 hours, and ellagic acid caused a 2.3-fold decrease in log 10 CFU/ml after 24 hours of exposure. Treatment with the MIC concentration of ellagic acid resulted in a 4-fold decrease in the bacterial population after 8 hours of incubation, and no viable cells were found after 24 hours. Treatment with 2MIC concentration drastically decreased the number of living *P. aeruginosa* cells and no living cells were observed after **8** hours of treatment. It was also observed that the reduction in bacterial population caused by ellagic acid depended on the concentration of ellagic acid and its exposure time.

http://dx.doi.org/10.22108/bjm.2023.137161.1532

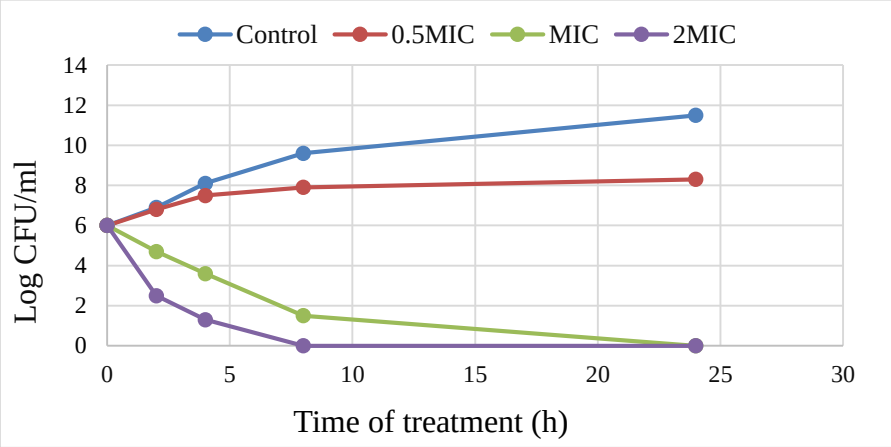

Figure 2. Effect of different concentrations of ellagic acid on the viability of *P. aeruginosa*

Investigation of the effect of ellagic acid on the killing time of *P. aeruginosa* under stress conditions

The killing time of ellagic acid against *P. aeruginosa* was measured under H_2O_2 stress conditions in the absence of ellagic acid (control) and in the presence of 1/2MIC and 1/4MIC concentrations of ellagic acid within 6 hours. Compared to the control, ellagic acid could increase the susceptibility of *P. aeruginosa* to stress conditions and further reduce the viability of the bacteria. Thus, after 4 hours of incubation, stress-induced bacteria treated with 1/2MIC ellagic acid showed a 3-fold decrease in log10CFU/mL compared to the control ($P<0.05$), and no viable cells were recovered after 6 hours of exposure. Under the same conditions, treatment with 1/4MIC concentration of ellagic acid resulted in a **3**.4 and **4**.7 fold decrease in log10CFU/mL ($P<0.05$) after 4 and 6 hours, respectively, compared to the control.

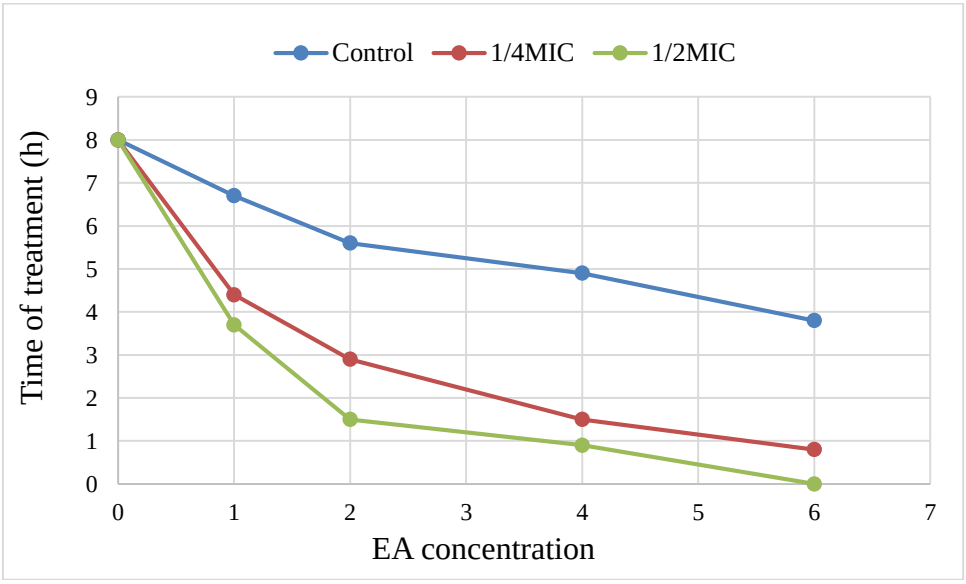

Figure **3**. The effect of different concentrations of ellagic acid (EA) on the survival of *P. aeruginosa* under oxidative stress conditions

Investigation of the effect of ellagic acid on the expression level of *rpo*S

Examination of *rpo*S gene expression in two isolates of *P. aeruginosa* treated with sub-MIC concentration of ellagic acid showed that ellagic acid significantly decreased the expression of the above gene compared to the control ($p<0.05$). In isolate 1 and isolate 2 treated with 500 µg/ml ellagic acid, the expression of the *rpo*S gene was downregulated by 0.22±0.04 and 0.36±0.02-fold, respectively (Figure 4**).**

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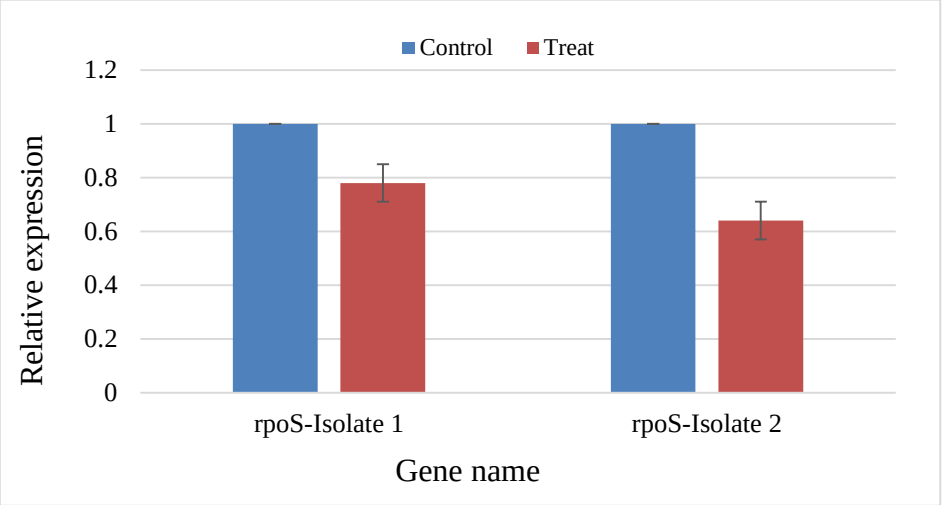

Figure 4. Changes in the expression level of the *rpo*S gene (reduced by 22% and 36%) in *P. aeruginosa* isolates treated with 1/2MIC concentration of ellagic acid.

Discussion

P. aeruginosa is a major cause of hospital-acquired infections, which are difficult to eradicate due to *P. aeruginosa's* low nutrient requirements, resistance to a wide range of temperatures , and resistance to antimicrobial agents and antibiotics. The emergence of resistance among major bacterial pathogens is recognized as a major threat to human health worldwide. Multi-drug resistant organisms have emerged not only in the hospital environment, but are now frequently identified in community settings, suggesting that reservoirs of antibiotic resistant bacteria exist outside the hospital (19). Empiric treatment of MDR/XDR *P. aeruginosa* often involves administering a traditional antipseudomonal β-lactam along with a second agent from a different antibiotic class (20). As *P. aeruginosa* can develop resistance through various mechanisms, there is an urgent need for a cost-effective alternative to develop a new generation of treatments for infectious diseases (20, 21). Natural products, especially plant-derived compounds, could be a potential source of new and effective herbal medicines to treat infections caused by drug-resistant bacteria (22). Particularly in developing countries, traditional herbal medicines have played an important role in the treatment of acute conditions such as indigestion, gastritis and peptic ulcers (23**)**. Previous research has shown that ellagic acid, the main polyphenol found in fruits, vegetables and nuts, has antimicrobial properties (24, 25). Ellagic acid also reduces the surface motility of *P. aeruginosa*, which is necessary for successful colonization of this bacterium (26). Incidentally the antipseudomonal activity and killing properties of ellagic acid have not been previously demonstrated.

In this study, ellagic acid showed an inhibitory effect on the growth of all MDR isolates of *P. aeruginosa*. The diameter of the inhibition zone produced by **5** mg of ellagic acid in the isolates tested varied between 8- 25 mm. The MIC of ellagic acid in 5 isolates tested varied between 250-2000 µg/ml. These different ranges of MIC values may be explained by differences in physiological and structural properties in clinical isolates of *P. aeruginosa.*

In the Time-killing assay, ellagic acid reduced the viability of *P. aeruginosa* at different times and this reduction in the bacterial population depended on the concentration of ellagic acid and the time of exposure of the bacteria to it. *P. aeruginosa* has a significant ability to survive under difficult and stressful conditions, making it difficult to control this bacterium in the hospital environment. In the present study, treatment of drug-resistant clinical strains of *P. aeruginosa* with ellagic acid increased their sensitivity to oxidative stress and decreased their viability. The viability of bacterial cells treated with ellagic acid decreased in a time- and dose-dependent manner. Our results confirm previous findings that ellagic acid reduced the expression of *rpoS* and increased the sensitivity of *P. aeruginosa* to oxidative (H_2O_2) stress (16). These findings suggest that treatment with ellagic acid and oxidative stress in the body have synergistic effects. The antibacterial effect of ellagic acid may be related to its action on the membrane of microorganisms (26). The ability of ellagic acid to form complexes with essential metals in bacterial cells is also responsible for its toxicity (26).

During infection, contact between *P. aeruginosa* and macrophages and neutrophils lead to activation of these cells and production of reactive oxygen species (ROS) such as hydrogen peroxide and

superoxide ions, which are lethal to the bacterial cell. *P. aeruginosa* survives oxidative stress by producing catalases (KatA and KatB) and superoxide dismutases (SODA and SODB) (**17**). A previous study demonstrated the effect of ellagic acid in reducing the activity of such antioxidant enzymes, including polyphosphate kinase (**16**). It has also been found that the downregulation of oxidative stress response genes, including the catalase genes *kat*A and *kat*B, is due to defective expression of the stationary phase sigma factor RpoS (**27**). Overexpression of *rpo*S also results in the restoration of antibiotic tolerance in *P. aeruginosa* (**17**). The significant decrease in the expression of *rpo*S, the downstream master stress response regulator, in isolates treated with sub-inhibitory concentrations of ellagic acid obtained in the present study may also be the reason for the decrease in the viability of bacterial cells after exposure to hydrogen peroxide. Similar research has elucidated the contribution of reactive oxygen species and oxidative stress to the antibacterial activity of ursolic acid against *Escherichia coli*, *P. aeruginosa* and *Staphylococcus aureus* (**28**).

Conclusion

The results of this study demonstrated the inhibitory effect of ellagic acid on the growth of *P. aeruginosa* isolates and increased their sensitivity to oxidative stress conditions. Our results suggest that ellagic acid has the potential to be used as a future alternative for the treatment of *P. aeruginosa* infections. However, conducting in vivo studies may clarify the possibility of its clinical application in controlling infections caused by drug-resistant isolates of *P. aeruginosa*. However, the results of the present study don't confirm that ellagic acid treatment reduces the tolerance of bacterial strains to oxidative stress in vivo, and further in vitro and in vivo studies are needed.

Acknowledgments

The authors would like to thank the Islamic Azad University, Rasht Branch, for their support.

Authors' Contributions

PT was involved in collecting samples, conducting experiments, and writing the manuscript. Study design, analyzing the results, and correcting of the manuscript were done by LA.

Competing Interests

The authors declare that there is no conflict of interest.

Funding

The authors didn't receive any financial support.

Ethical Issues

Not included.

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